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(54) Title: NEW *CANDIDA ALBICANS* KRE9 AND USES THEREOF

(57) Abstract

The present invention relates to an isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein the gene is referred to as *CaKRE9*, wherein the sequence of the DNA is as set forth in Fig. 1. The present invention relates to antifungal *in vitro* and *in vivo* screening assays for identifying compounds which inhibit the synthesis, assembly and/or regulation of β1,6–glucan. There is also disclosed an *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient.

NEW *CANDIDA ALBICANS KRE9* AND USES THEREOF

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a novel gene, *CaKRE9*, isolated in the yeast pathogen, *Candida albicans*, that is a functional homolog of the *S. cerevisiae KRE9* gene and which is essential for cell wall glucan synthesis, and to novel antifungal screening assays.

(b) Description of Prior Art

Fungi constitute a vital part of our ecosystem but once they penetrate the human body and start spreading they cause infections or "mycosis" and they can pose a serious threat to human health. Fungal infections have dramatically increased in the last 2 decades with the development of more sophisticated medical interventions and are becoming a significant cause of morbidity and mortality. Infections due to pathogenic fungi are frequently acquired by debilitated patients with depressed cell-mediated immunity such as those with human immunodeficiency virus (HIV) and now also constitute a common complication of many medical and surgical therapies. Risk factors that predispose individuals to the development of mycosis include neutropenia, use of immunosuppressive agents at the time of organ transplants, intensive chemotherapy and irradiation for hematopoietic malignancies or solid tumors, use of corticosteroids, extensive surgery and prosthetic devices, indwelling venous catheters, hyperalimentation and intravenous drug use, and when the delicate balance of the normal flora is altered through antimicrobial therapy.

The yeast genus *Candida* constitutes one of the major groups that cause systemic fungal infections and the five medically relevant species which are most

often recovered from patients are *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*.

5   Much of the structure of fungal and animal cells along with their physiology and metabolism is highly conserved. This conservation in cellular function has made it difficult to find agents that selectively discriminate between pathogenic fungi and their human hosts, in the way that antibiotics do between bacteria and man. Because of this, the common antifungal drugs,

10   like amphotericin B and the azole-based compounds are often of limited efficacy and are frequently highly toxic. In spite of these drawbacks, early initiation of antifungal therapy is crucial in increasing the survival rate of patients with disseminated candidiasis.

15   Moreover, resistance to antifungal drugs is becoming more and more prominent. For example, 6 years after the introduction of fluconazole, an alarming proportion of *Candida* strains isolated from infected patients have been found to be resistant to this drug and this is

20   especially the case with vaginal infections. There is thus, a real and urgent need for specific antifungal drugs to treat mycosis.

The fungal cell wall: a resource for new antifungal targets

25   In recent years, we have focused our attention on the fungal extracellular matrix, where the cell wall constitutes an essential, fungi-specific organelle that is absent from human/mammalian cells, and hence offers an excellent potential target for specific antifungal

30   antibiotics. The cell wall of fungi is essential not only in maintaining the osmotic integrity of the fungal cell but also in cell growth, division and morphology. The cell wall contains a range of polysaccharide polymers, including chitin, β-glucans and O- and N-linked

35   mannose sidechains of glycoproteins. β-glucans, homopolymers of glucose, are the main structural component

of the yeast cell wall, and constitute up to 60% of the
dry weight of the cell wall. Based on their chemical
linkage, two different types of polymers can be found:
β1,3-glucan and β1,6-glucan. The β1,3-glucan is the

5 most abundant component of the cell wall and it con-
tains on average 1500 glucose residues per molecule.
It is mainly a linear molecule but contains some 1,6-
linked branchpoints. The β1,6-glucan is a smaller and
highly branched molecule comprised largely of 1,6-

10 linked glucose residues with a small proportion of 1,3-
linked residues. The average size of β1,6-glucan is
approximately 400 residues per molecule. The β1,6-glu-
can polymer is essential for cell viability as it acts
as the "glue" covalently linking glycoproteins and the

15 cell wall polymers β1,3-glucan and chitin together in a
crosslinked extracellular matrix.

It would be highly desirable to be provided with
the identification and subsequent validation of new
cell wall related targets that can be used in specific

20 enzymatic and cellular assays leading to the discovery
of new clinically useful antifungal compounds.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide
the identification and subsequent validation of a new

25 target that can be used in specific enzymatic and cel-
lular assays leading to the discovery of new clinically
useful antifungal compounds.

Although a gene involved in the cellular growth

30 of *S. cerevisiae* was identified, there are no certain-
ties that there would be a homolog in *Candida albicans*
or if present that it would have the same function.

In accordance with the present invention a gene
was isolated, *CaKRE9*, in the yeast pathogen, *Candida*

35 *albicans*, that is a functional homolog of the *S. cere-
visiae KRE9* gene and which is essential for cell wall

glucan synthesis. The gene is not found in humans and when it is inactivated in *C. albicans*, the cell cannot survive when grown on glucose, thus, validating it as a wholly new target for antifungal drug discovery.

Using the gene of the present invention, we intend to utilize novel drug screening assays for which we possess all the genetic tools.

In accordance with the present invention there is provided an isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein the gene is referred to as *CaKRE9*, wherein the sequence of the DNA is as set forth in Fig. 1.

In accordance with the present invention there is also provided an antifungal screening assay for identifying a compound which inhibits the synthesis, assembly and/or regulation of β1,6-glucan, which comprises the steps of:

a)   synthesizing β1,6-glucans *in vitro* from activated sugar monomer/polymer and specific β1,6-glucan synthetic proteins;

b)   subjecting step a) to a high throughput compound screen determining absence or presence of β1,6-glucan, wherein absence of β1,6-glucan is indicative of an antifungal compound.

In accordance with the present invention there is also provided an *in vivo* antifungal screening assay for identifying compounds which inhibit the synthesis, assembly and/or regulation of β1,6-glucan, which comprises the steps of:

a)   separately cultivating a mutant yeast strain lacking one gene for synthesis of β1,6-glucans and a wild type yeast strain with activated sugar monomer/polymer UDP-glucose;

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   b)   subjecting both yeast strains of step a) to the
        screened compound and determining if the com-
        pound selectively inhibits growth of wild type
        strain which is indicative of an antifungal com-
5   pound.

        In accordance with the present invention there
is also provided an *in vitro* method for the diagnosis
of diseases caused by fungal infection in a patient,
which comprises the steps of:

10   a)   obtaining a biological sample from the patient;
     b)   subjecting the sample to PCR using a primer pair
          specific for *CaKRE9* gene, wherein a presence of
          the gene is indicative of the presence of fungal
          infection.

15   In accordance with the present invention, the
gene is *CaKRE9*.

        In accordance with the present invention there
is also provided an *in vitro* method for the diagnosis
of diseases caused by fungal infection in a patient,
20   which comprises the steps of:

     a)   obtaining a biological sample from the patient;
     b)   subjecting the sample to an antibody specific
          for *CaKre9p* antigen, wherein a presence of the
          antigen is indicative of the presence of fungal
25   infection.

        In accordance with one embodiment of the present
invention, the fungal infection may be caused by *Can-
dida*.

        In accordance with the present invention there
30   is also provided the use of at least one of *KRE9* and
CaKre9 nucleic acid sequences and fragments thereof as
a probe for the isolation of *KRE9* homologs in all
fungi.

        For the purpose of the present invention the
35   following terms are defined below.

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The term a "mutant yeast strain" is intended to mean any yeast strain lacking one gene for synthesis of β1,6-glucan, such as *KRE9* and homologs thereof.

The term a "wild type yeast strain" is intended
5 to mean any yeast strain containing the *KRE9* gene or a homolog thereof or a plasmid overexpressing the *KRE9* gene or a homolog thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

10 Fig. 1 illustrates the complete nucleotide and predicted amino acid sequence of CaKRE9 (SEQ ID NO:1-2).

Fig. 2 illustrates the comparison of the sequence of Kre9p from *Candida albicans* (SEQ ID NO:2)
15 and Kre9p (SEQ ID NO:3) and Knh1p (SEQ ID NO:4) from *Saccharomyces cerevisiae*;

Fig. 3 illustrates the *CaKRE9*-dependent effect on the growth (A) and Killer phenotype (B) of *kre9Δ* null mutants;

20 Fig. 4A illustrates the schematic representation of the strategy for disruption of the *Candida albicans* *KRE9* gene;

Fig. 4B illustrates the Southern blot verification of the correct integration of the *hisG-URA3-hisG*
25 disruption module into the *CaKRE9* gene and proper *CaURA3* excision after 5-FOA treatment; and

Fig. 5 illustrates the quantification of β1,6-Glucan levels of different *Candida albicans* strains.

DETAILED DESCRIPTION OF THE INVENTION

30 In accordance with the present invention, the synthesis and the assembly of the cell wall polymer β1,6 glucan which plays a central role in the organization of the yeast cell wall and which is indispensable
35 for cell viability were extensively studied. Although

the biochemistry of β1,6 glucosylation is incompletely understood, a genetic analysis of genes required for 1,6 synthesis has been performed in *Saccharomyces cerevisiae*, and has identified many genes required for this

5   process. These encode products acting in the endoplasmic reticulum, the Golgi complex and at the cell surface.

In accordance with the present invention a novel gene was identified, *KRE9*, whose product is required

10 for the synthesis of β1,6 linked glucans (Brown JL. et al. (1993) *Molecular & Cellular Biology* **13**:6346-6356). *KRE9* appears to be a fungal specific gene, as it is absent from animal lineages based on data base searches of the *Caernorhabditis elegans*, mouse and *Homo sapiens*

15 genomes and it also appears to be absent from the plant, bacterial and archaebacterial lineages.

KRE9 and its homolog KNH1

KRE9 encodes a 30-kDa secretory pathway protein involved in the synthesis of cell wall β1,6 glucan

20 (Brown JL. et al. (1993) *Molecular & Cellular Biology* **13**:6346-6356). Disruption of *KRE9* in *S. cerevisiae* leads to serious growth impairment and an altered cell wall containing less than 20% of the wild-type amount of β1,6 glucan. Analysis of the glucan material

25 remaining in a *kre9* null mutant indicated a polymer with a reduced average molecular mass (Brown JL. et al. (1993) *Molecular & Cellular Biology* **13**:6346-6356). The *kre9* null mutants also displayed several additional cell-wall-related phenotypes, including an aberrant

30 multiple budded morphology, a mating defect, and a failure to form projections in the presence of alphafactor. Antibodies generated against Kre9p detected an *O*-glycoprotein of approximately 55 to 60 kDa found in the extracellular medium of a strain overproducing

Kre9p, indicating it is normally localized at the cell surface.

In the yeast genome a *KRE9* homolog was recently found, *KNH1*, whose product, Knh1p, shares 46% overall identity with Kre9p (Dijkgraaf GJ. et al. (1996) *Yeast* **12**:683-692). Disruption of the *KNH1* locus has no effect on growth, killer toxin sensitivity or β1,6-glucan levels. Overexpression of *KNH1* suppressed the severe growth defect of a *kre9* null mutant and restored the level of alkali-insoluble β1,6-glucan to almost wild type levels. When overproduced, Knh1p, like Kre9p, can be found in the extracellular culture medium as an O-glycoprotein, and is likely also a cell surface protein under conditions of normal expression. The disruption of both *KNH1* and *KRE9* is lethal. Transcription of *KNH1* is carbon-source and *KRE9* dependent. The severe growth defect of a *kre9Δ* null mutant observed on glucose can be partially restored when galactose becomes the major carbon source. Transcription of the *KNH1* gene is normally low in wild type cells grown on glucose but increases approximately five fold in galactose grown cells, where it partially compensates for the loss of Kre9p and allows partial suppression of the slow growth phenotype of *kre9Δ* cells. These results suggest that *KRE9* and *KNH1* are specialized *in vivo* to function under different environmental conditions (Dijkgraaf GJ. et al. (1996) *Yeast* **12**:683-692).

The essential nature of the *KRE9/KNH1* gene pair, and the putative extracellular location of their gene products make these proteins a new and potentially valuable target for antifungal compounds that need not enter the fungal cell.

β1,6-glucan in pathogenic fungi

The yeast *Saccharomyces cerevisiae*, although not a pathogen, is a proven model organism for pathogenic

fungi as it is closely related taxonomically to opportunistic pathogens like the dimorphic yeast *Candida albicans*. The composition of the cell wall of *C. albicans* resembles that of *S. cerevisiae* in containing β1,3- and β1,6-glucans, chitin, and mannoproteins (Mio, T. et al., *J. Bacteriol.* 179:2363-2372 Analyses of the *Candida albicans* genes involved in extracellular matrix assembly are limited but indicate that the proteins responsible for synthesis of the polymers often resemble those found in the more extensively studied yeast, *Saccharomyces cerevisiae*. The β1,6 glucosylation of proteins appears to be widespread among fungal groups, and the polymer varies in abundance between fungal species. In *C. albicans* this polymer is particularly abundant, comprising approximately half of the alkali insoluble glucan. Comparative studies with *C. albicans* have so far identified three genes involved in β1,6 glucosylation based on their relatedness to those in *S. cerevisiae*, indicating that synthesis of this polymer is functionally conserved and essential for the growth of *Candida albicans*.

Isolation of the *CaKRE9* gene

In order to validate *KRE9* as a possible new antifungal target, we have examined if genes related to *S. cerevisiae KRE9* were present in *C. albicans*. Using complementation of the *S. cerevisiae kre9* mutant phenotype as a screen, we have isolated a *C. albicans* gene that encodes a protein similar to the *S. cerevisiae KRE9* gene product.

CaKRE9 was identified by a plasmid shuffle approach as a gene being able to restore the slow growth of a *Saccharomyces cerevisiae kre9::HIS3* disrupted strain. A diploid strain heterozygous for a *kre9::HIS3* deletion was transformed with a centromeric *LYS2*-based pRS317 vector containing a wild type copy of

the *S. cerevisiae KRE9* gene.   Transformants were
selected by prototrophic growth on minimal media,
sporulated and a haploid *kre9::HIS3* strain containing a
plasmid-based copy of *KRE9* was obtained by tetrad dis-
5 section and spore progeny analysis.   This strain was
shown to possess wild type growth and killer toxin sen-
sitivity ·and was subsequently transformed with a *Can-
dida albicans* genomic library contained within the mul-
ticopy YEp352-plasmid harboring the *URA3* gene as a
10 selectable marker.   In order to screen for plasmids
that could restore growth to a *kre9::HIS3* mutant, about
20,000 His3⁺ Lys2⁺ Ura3⁺ cells were replica plated on
minimal medium containing α-aminoadipate as a primary
nitrogen source to select for cells that have lost the
15 *LYS2* plasmid-based copy of *KRE9* but are still able to
grow, indicating that a copy of the complementing
CaKRE9 gene could be present in such growing cells.
These cells were further tested for loss .of the pRS317-
KRE9 plasmid by failure to grow on medium lacking
20 lysine.   YEp352-based *Candida albicans* genomic DNA was
recovered from cells that grew in the presence of
lysine but did not grow in its absence.   Upon
retransformation in yeast, only 2 different genomic
inserts were able to partially restore growth of the
25 *kre9::HIS3* haploid strain.   DNA from both inserts were
sequenced.
         The *CaKRE9* gene was contained in only one of the
C. albicans clones.   Complete sequencing of the 8-kb
fragment containing the *CaKRE9*   gene revealed an open
30 reading frame of 813 bp encoding a 29-kDA secretory
protein of 271 amino acid residues (see Fig. 1).   As is
the case with Kre9p and Knh1p (Brown JL. et al. (1993)
Molecular & Cellular Biology **13**:6346-6356; Dijkgraaf
GJ. et al. (1996) *Yeast* **12**:683-692), the hydrophobic N-
35 terminal region of CaKre9p comprises an eukaryotic sig-

nal sequence, with the most likely cleavage site occurring between amino acid residues 21 and 22. CaKre9p shares 43% overall identity with Kre9p and 32% with Knh1p (see Fig. 2). The amino acid residues are shown
5   in single-letter amino acid code. Sequences were aligned with gaps to maximize homology. Dots represent a perfect match between all sequences while a vertical slash indicates conservative substitution at a given position. The most conserved region between the 3 pro-
10 teins encompasses a large part of the central region and most of the C-terminal portion, with the N-terminal part being largely unique to each protein. Kre9p, Knh1p and CaKre9p share a high proportion of serine and threonine residues (26%), potential sites for O-glyco-
15 sylation, a modification known to occur on Kre9p and Knh1p, and characteristic of many yeast cell surface proteins. In addition, all 3 proteins have lysine and arginine rich C-termini and lack potential N-linked glycosylation sites.
20   The functional capacity of CaKre9p was assessed in *Saccharomyces cerevisiae* by measuring its ability to restore the growth and killer toxin sensitivity of a *kre9* null mutant. Firstly, the YEp352-based *Candida albicans* genomic DNA containing the *CaKRE9* gene was
25 transformed into a diploid strain of *S. cerevisiae* heterozygous for a *kre9::HIS3* deletion, sporulated and a haploid *kre9::HIS3* strain containing a plasmid-based copy of *CaKRE9* was obtained from spore progeny following tetrad dissection. As can be seen in Fig. 3A, a
30 strain harboring the *CaKRE9* gene grows at a slower rate than a wild type strain or the mutant strain harboring a copy of *KRE9* but significantly faster than the *kre9* null mutant which has a severe growth phenotype. Secondly, the haploid *kre9* strain carrying the *CaKRE9* was
35 submitted to a killer toxin sensitivity assay (Fig.

3B). K1 killer yeast strains secrete a small pore-forming toxin that requires an intact cell wall receptor for function. *KRE9* null mutations lead to a considerable decrease in the level of β1,6-glucan disrupt-

5 ing the toxin receptor (Brown JL. et al. (1993) *Molecular & Cellular Biology* **13**:6346-6356), leading to killer resistance and showing no killing zone in the assay. The killer phenotype of the *kre9* mutant allowed a test of possible suppression by CaKre9p. Overexpression of

10 *CaKRE9* in the *S. cerevisiae* haploid strain carrying a disrupted copy of *KRE9* partially suppressed the killer resistance phenotype (Fig. 3B).

These results imply that Kre9p and CaKre9p both play very similar roles in β1,6-glucan assembly in *S.*

15 *cerevisiae* and *C. albicans*.

Disruption of the *CaKRE9* gene

Experimental strategy:

The gene disruption was performed by the *URA* blaster protocol using the *hisG-CaURA3-hisG* module. A

20 1.6-kb DraI DNA fragment containing the *CaKRE9* gene was subcloned from the original insert into the SmaI site and the blunted XbaI site (treated with the Klenow fragment of DNA polymerase I) of YEp352 (see Fig. 4A). Extracted genomic DNAs are from : CAI4 wild type cells

25 (lane 1), *CaKRE9/Cakre9::hisG-URA-hisG* heterozygous mutant (lane 2), *CaKRE9/Cakre9::hisG* heterozygous mutant obtained after 5-FOA treatment (lane 3) and *Cakre9/Cakre9::hisG-URA-hisG* homozygous null mutant which is able to grow only when galactose is used as

30 the sole source of carbon.

The *CaKRE9* gene was disrupted by deleting a 485 bp BstxI-BamHI fragment of the open reading frame and replacing it by a 4.0 kb BglII/BamHI fragment carrying the *hisG-URA3-hisG* module from plasmid pCUB-6 (see

35 Fig. 4A). The sticky ends were enzymatically treated to

accommodate the ligation. This disruption plasmid was digested by HindIII and KpnI, precipitated with ethanol and sodium acetate and 100 μg of the 5.2 kb-disruption fragment was transformed into CAI4 *Candida albicans*
5   cells by the lithium acetate method.

Putative heterozygous disruptants were selected on minimal medium carrying glucose or galactose as carbon sources but lacking uracil. In preparation for a second round of gene disruption, the *CaURA* gene was
10 excised using a 5-FOA selection. The second round of transformation was performed in the same way as the primary one.

The accurate integration of the *hisG-CaURA3-hisG* cassette into the *CaKRE9* gene and its excision from
15 genomic DNA was verified by Southern hybridization using 3 different probes:

(1) a 405-bp fragment from *C. albicans* genomic DNA containing coding and 3' flanking sequences of *CaKRE9*;

(2) a 783 bp DNA fragment obtained by PCR and covering
20 the entire *CaURA3* coding region; and

(3) a 898 bp fragment amplified by PCR that encompasses the whole of the *Salmonella typhimurium hisG* gene (see Fig. 4B).

All genomic DNAs were digested with the BamHI
25 and SalI restriction enzymes.

Results:

In the first round of transformation where transformants were selected on glucose containing plates, the Southern blotting results revealed that the
30 *hisG-CaURA3-hisG* module correctly integrated into the *Candida albicans* KRE9 gene (see Fig. 4). When genomic DNA of putative heterozygous *CaKRE9* disruptions was digested with the SalI and BamHI restriction enzymes and probed with the *CaKRE9* 405-bp SalI-BstXI DNA frag-
35 ment along with the *hisG* and the *CaURA3* probes, 2

expected bands could be detected (see Fig. 4B, lane 2,
for representative result): a 773 bp band corresponding
to the wild type gene that could only be detected by
the *CaKRE9* probe and a 4318 bp diagnostic band,
5   revealed by all 3 probes, indicating successful disrup-
tion of one copy of the *CaKRE9* gene. After removal of
the *CaURA3* using 5-FOA, the 773 bp wild type band could
still be visualized but the disrupted band from which
the *CaURA3* was excised shifted to an anticipated 1428
10   bp when probed with the *CaKRE9* and *hisG* probes but not
with the *CaURA3* probe (see Fig. 4B, lane 3).

        In order to assess if the *CaKRE9* gene is essen-
tial in *C. albicans*, a second round of disruptions was
undertaken in the heterozygous strain where the *CaURA3*
15   gene was eliminated. However, in view of the nature of
the carbon source regulation of the *KRE9/KNH1* pair in
S. cerevisiae, the second round of transformation was
executed using both glucose and galactose as carbon
sources.   32 Ura$^+$ colonies from the glucose plated
20   transformation were analyzed by Southern blot hybridi-
zation using the 3 different probes and only yeast
cells heterozygous at the *CaKRE9* locus could be found.
The absence of the expected homozygous double disrup-
tion among the transformants is consistent with the
25   fact that *CaKRE9* is an essential gene in *C. albicans*
when glucose is the sole carbon source. Demonstration
of *CaKRE9* as an essential gene under these conditions
validates the *CaKRE9* gene product as a therapeutic tar-
get in *Candida albicans*.

30   The population of transformants growing on
galactose was heterogeneous with large and small sized
colonies occurring. As a first assessment of a possi-
ble carbon source dependence, a total of 26 colonies of
different sizes were plated from galactose to glucose.
35   Among the smaller ones, 8 did not grow on glucose, sug-

gesting that they could be homozygous disruptants. Southern blot hybridizations were performed on these 8 transformants and they were shown to be homozygous disruptants for the *CaKRE9* locus: one copy corresponded to the disrupted gene in which *CaURA3* has been removed (1428 bp) and the second one represented the inactivation of the remaining wild type copy by the *hisG-caURA3-hisG* module (4318 bp; Fig. 4B, lane 4). Thus a homozygous disruption of *kre9* in *C. albicans* is lethal when glucose constitutes the exclusive carbon source. Further, it should be appreciated that glucose is the main source of carbon of human beings.

β1,6-glucan analysis of *C. albicans* *CaKRE9* mutants

Experimental strategy:

Yeast total-cell protein extracts were prepared from exponentially growing cultures by cell lysis with glass beads. Cellular extracts were standardized for total cellular protein and equivalent amounts of protein were alkali extracted (0.75M NaOH final 1h, 75°C). The alkali soluble fractions were then spotted onto nitrocellulose and immunoblots were carried out. Briefly, blots were treated in TBST buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween™ 20, containing 5% non fat dried milk powder) and subsequently incubated with affinity purified rabbit anti-β1,6-glucans antibodies (prepared as described Montijn, R.C. et al. (1994) *J. Biol. Chem.* **296**:19338-19342) in the same buffer. After antibody binding, membranes were washed in TBST and a second antibody directed against rabbit immunoglobulins and conjugated with horseradish peroxidase, was then added. The blots were again washed and whole cell β1,6 glucans detected using an enhanced chemiluminescence procedure.

Results

In order to directly measure the effect of inac-
tivating *CaKRE9* on β1,6-glucan synthesis and assembly,
a specific rabbit anti-β1,6-glucan antiserum was raised
against BSA-coupled pustulan (a commercially available
5   β1,6 glucan), affinity purified, and used to detect
antigen-antibody complexes by Western blotting of total
cell protein extracts of different yeast strains grown
on galactose. As expected, wild type cells yielded a
strong β1,6-glucan signal (see Fig. 5). The affinity
10 purified Ab detected about a quarter of the glucan in
the *C. albicans* heterozygous *Δcakre9* whereas no β1,6-
glucan could be detected from a *C. albicans* homozygous
Δcakre9 disruptant grown on galactose (Fig. 5).

Discussion

15   The essential nature of the *KRE9* gene in *C.
albicans*, and the possible extracellular location of
its gene product make this protein a new and poten-
tially valuable target for antifungal compounds that
need not enter the fungal cell. The precise role of
20 Kre9p in β-glucan synthesis remains to be precisely
determined but does not prevent the establishment of a
antifungal drug screening assay

The present invention will be more readily un-
derstood by referring to the following examples which
25 are given to illustrate the invention rather than to
limit its scope.

<u>EXAMPLE I</u>

In vitro **screening method for specific antifungal**
agents (enzymatic-based assay)

30   The primary objective is to identify novel com-
pounds inhibiting the synthesis, assembly and/or regu-
lation of β1,6-glucans. This enzymatic assay would
utilize some of the gene products (*KRE*) involved in
β1,6-glucan synthesis, including using an *in vitro*
35 assay for CaKre9p. Using specific reagents such as an
antibody to β1,6-glucan, and a specific glucanase for

the polymer, the approach is to synthesize the polymer
in vitro from the activated sugar monomer UDP-glucose.
This task can be accomplished by existing methodologies
such as the production of large amounts of each protein
5   and by the availability of genetic tools, such as the
ability to delete or overexpress gene products that are
involved in synthesis of this and the other major poly-
mers. Once the assay has been established it will per-
mit the screening of possible compounds that inhibit
10   steps in the synthesis of this essential polymer. When
such inhibitors will be found, they will then be evalu-
ated as candidates for specific antifungal agents.

The effects of such compounds on β1,6-glucan
levels may be directly measured using the anti-β1,6-
::   glucan antibody. This approach can be used on all type
of fungi and can be adapted to a high throughput immu-
noassay to find β1,6-glucan inhibitors.

EXAMPLE II

20   *In vivo* **screening method for specific antifungal agents**
(cellular-based assay)

Yeast strains possessing or lacking β1,6-glucans
permit a differential screen for compounds inhibiting
synthesis of this cell wall polymer. Specifically, an
25   antifungal drug screen can be devised based on a whole-
cell assay in which the fungal-specific CaKre9p would
be targeted.

The strains that may be used in accordance with
the present invention include, without limitation, any
30   yeast strain mutant for *CaKRE9* and homologs thereof
disrupted strain, conditional mutants, overexpression
strains and suppressed disrupted strains.

Compounds can be tested for their ability to
inhibit growth or kill a wild type *C. albicans* strain
35   while having no effect on a *Cakre9* suppressor strain.
In addition, compounds leading to hypersensitivity in a

CaKRE9 deletion will also be of value as candidate antifungal drugs. The finding of new antifungal compounds will be greatly simplified by these types of screens. The direct scoring on cells of the level of

5   efficacy of a particular compound (natural product extracts, pure chemicals...) alleviates the costly and labor intensive establishment of an *in vitro* enzymatic assay. The availability of genetic tools, such as the ability to delete or overexpress gene products that are

10   involved in synthesis of this and the other major polymers will permit the establishment of this new screening method. When such inhibitors will be found, they will then be evaluated as candidates for specific antifungal agents.

15

EXAMPLE III

The use of *CaKRE9* in the diagnosis of fungal infection

Detection based on PCR

Candida spp. and other pathogenic fungi are tradi-

20   tionally identified by morphological and metabolic characteristics and often this require days to weeks to isolate on culture from a patient's sample. Identification is time-consuming and often unreliable and this impedes the selection of antimicrobial agents in cases

25   in which species identification of the organism is necessary. Moreover, culture-based diagnostic methods are not within the scope of many routine microbiology laboratories and are frequently limited to detection of pathogenic organisms in patients at an advanced stage

30   of disease or even at autopsy. The detection of disseminated *Candida* mycosis is an area where there is an urgency for new sophisticated techniques of identification. Polymerase Chain Reaction (PCR) based tests to establish the presence of a fungal infection are at

35   this point highly desirable for laboratory diagnosis and management of patients with serious fungal dis-

eases. The *CaKRE9* gene is fungi specific and could be
used to develop new diagnostic procedures of mycosis
based on the PCR. Such diagnostic tests would be pre-
dicted to be highly sensitive and specific. Ulti-
5   mately, simple kits permitting the diagnosis of fungal
infections will be sold to hospitals and specialized
clinics. Current trends in the hospital microbiology
laboratories indicate that there will be a considerable
future increase in use of the PCR as a diagnostic tool.

10   **Detection based on anti-CaKre9p antibodies**

       CaKre9p is thought to be localized at the cell
surface and as such could be detected as a circulating
candidal antigen by an enzyme-linked immunoabsorbent
assay (ELISA) detection kit based on antibodies
15   directed against CaKre9p. Antibodies directed against
CaKre9p could allow levels of specificity and sensitiv-
ity high enough to permit commercialization of a diag-
nostic kit.

20   **EXAMPLE IV**

                The use of Kre9p in all fungi

       Isolation and use of functional homologs of
KRE9/CaKRE9 from all fungi. Most fungi have β1,6-glu-
cans and likely have *KRE9* homologs in their genome.
25   The *kre9* mutant can allow isolation of similar genes by
functional complementation from other pathogenic fungi
as what was done to isolate *CaKRE9*. *KRE9* could also
serve as a probe to isolate by homology *KRE9* homologs
from other yeasts. In addition, Kre9p allows isolation
30   of homologs in other species by the techniques of
reverse genetics where antibodies raised against Kre9p
could be used to screen expression libraries of patho-
genic fungi for expression of KRE9 homologs that would
immunologically cross react with antibodies raised
35   against *S. cerevisiae KRE9* and *C. albicans CaKRE9*.

These putative *KRE9* homologs in these pathogenic fungi could serve as targets for potential new antifungals.

Other methods are used to find proteins which interact with Kre9p and homologs thereof, such as two-hybrid, co-immunoprecipitation and chromatography using an activated Kre9p matrix.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1.   An isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein said gene is referred to as *CaKRE9*, wherein the sequence of said DNA is as set forth in Fig. 1 and yeast homologs thereof.

2.   An antifungal screening assay for identifying a compound which inhibits the synthesis, assembly and/or regulation of β1,6-glucan, which comprises the steps of:

   a)   synthesizing β1,6-glucan *in vitro* from activated sugar monomer/polymer and specific β1,6-glucan synthetic proteins;

   b)   subjecting step a) to a high throughput compound screen determining concentration of β1,6-glucan, wherein reduction in β1,6-glucan is indicative of an antifungal compound.

3.   The antifungal screening assay of claim 2, wherein said β1,6-glucan is absent.

4.   An *in vivo* antifungal screening assay for identifying compounds which inhibit the synthesis, assembly and/or regulation of β1,6-glucans, which comprises the steps of:

   a)   separately cultivating a mutant yeast strain lacking one gene for synthesis of β1,6-glucans and a wild type yeast strain with activated sugar monomer/polymer UDP-glucose;

   b)   subjecting said both yeast strains of step a) to the screened compound and determining if said compound selectively inhibits growth of wild

type strain which is indicative of an antifungal compound.

5.   The method of claim 3, wherein said gene is *CaKRE9*.

6.   An *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, which comprises the steps of:
   a) obtaining a biological sample from said patient;
   b) subjecting said sample to PCR using a primer pair specific for *CaKRE9* gene, wherein a presence of said gene is indicative of the presence of fungal infection.

7.   The method of claim 6, wherein said fungal infection is caused by *Candida*.

8.   An *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient, which comprises the steps of:
   a) obtaining a biological sample from said patient;
   b) subjecting said sample to an antibody specific for *CaKre9p* antigen, wherein a presence of said antigen is indicative of the presence of fungal infection.

9.   The method of claim 8, wherein said fungal infection is caused by *Candida*.

10.   The use of at least one of *KRE9* and *CaKre9* nucleic acid sequences and fragments thereof as a probe for the isolation of KRE9 homologs.

FIG-1A

```
                                                                      17
  Met Arg Gln Phe Gln Ile Ile Leu Ile Ser Leu Val Val Ser Ile Ile Arg
1 ATG AGA CAA TTT CAA ATC ATA TTA ATT TCC CTT GTT GTT TCC ATA ATA AGA

                                                                      34
   Cys Val Ala Asp Ile Thr Ser Ala Ser Gly Ser Ser Asp Ser Gly Glu Phe
52 TGT GTT GCA GAT ATC ACA TCA GCA TCT GGA TCA AGT GAT TCA GGA GAA TTT

                                                                       51
    Ser Gly Ser Ser Asp Ser Pro Lys Ser Asp Ser Lys Ser Pro Asp Ser Asp
103 TCT GGT AGT TCT GAT AGT CCT AAG TCA GAC AGT AAA TCA CCG GAT TCA GAC

                                                                       68
    Asp Ser Thr Gly Pro Thr Gly Pro Thr Asp Gly Ile Ala Gly Tyr Ile Ser
154 GAT TCA ACT GGA CCT ACT GGA CCT ACA GAT GGA ATT GCA GGT TAC ATT TCT

                                                                       85
    Leu Cys Thr Gly Pro Thr Gly Gln Ile Gly Lys Ser Asp Ile Asp Pro Leu
205 TTA TGT ACT GGA CCT ACT GGA CAG ATC GGG AAA TCA GAT ATT GAT CCA TTA

                                                                       102
    Val Lys Asn Glu Ala Ala Ile Gly Lys Ser Lys Ser Ile Ile Pro Pro Gln
256 GTC AAG AAC GAA GCT GCA ATT GGA AAA TCT AAA TCT ATT ATT CCC CCC CAG

                                                                       119
    Asn Ser Val Pro Asn Gly Tyr Tyr Tyr Val Thr Thr Phe Ser Val Phe Thr
307 AAC TCA GTA CCT AAT GGT TAT TAC TAT GTT ACA ACT TTC TCT GTT TTC ACT

                                                                       136
    Asn Gly Thr Thr Ile His Tyr Ser Pro Arg Phe Lys Leu Thr Thr Gly Met
358 AAT GGA ACC ACT ATT CAT TAT TCA CCA CGT TTC AAA TTG ACT ACT GGT ATG
```

```
          Ser Gly Pro Thr Ala Thr Leu Asp Val Thr Glu Thr Gly Ser Val Pro Ala
409       TCT GGT CCA ACT GCC ACT TTA GAT GTC ACC GAA ACA GGA TCG GTG CCA GCG   153

          Asp Gln Ala Ser Gly Phe Asp Thr Ala Thr Tyr Ala Asp Ser Lys Ser Phe
460       GAT CAA GCT TCA GGA TTT GAT ACT GCA ACT TAC GCC GAC TCC AAA TCT TTC   170

          Thr Val Pro Tyr Thr Leu Gln Thr Gly Lys Thr Arg Tyr Ala Pro Met Gln
511       ACA GTT CCA TAT ACC CTA CAA ACA GGG AAG ACC AGA TAC GCA CCA ATG CAA   187

          Met Gln Pro Gly Thr Lys Val Thr Ala Thr Ala Trp Ser Met Lys Phe Pro
562       ATG CAA CCA GGT ACC AAA GTG ACT GCT ACA GCT TGG AGT ATG AAG TTC CCA   204

          Thr Ser Ala Val Thr Tyr Ser Thr Ile Thr Pro Gly Thr Pro Asn Val Ala
613       ACT AGT GCT GTT ACT TAT TCA ACT ATT ACC CCA GGC ACA CCA AAT GTG GCC   221

          Ser Thr Ile Thr Pro Val Ala Pro Tyr Trp Pro Thr Tyr Ser Glu Arg Val
664       TCT ACT ATT ACC CCA GTT GCT CCA TAT TGG CCT ACA TAC TCT GAA CGA GTG   238

          Ala Ser Val Ala Pro Tyr Trp Pro Thr Tyr Trp Pro Ala Ser Glu Arg Val
715       GCT AGT GTT GCT CCA TAT TGG CCT ACA TAC TGG CCT GCC AGT GAA CGA GTG   255

          Ser Lys Ala Thr Ile Ser Ala Thr Ala Thr Lys Arg Arg Trp Leu Asp ***
766       AGT AAG GCT ACA ATT AGT GCT ACA GCT ACA AAG AGA AGA TGG TTG GAT TGA   271
```

FIG. 1B

```
                    10        20        30        40        50        60
                    |         |         |         |         |         |
CaKre9p   MRQFQ-IILISLVVSIIRCVVADVDITSPKSGETFSGSSGSASI-KITWDDSDDSDSPKSLDN
Kre9p     MRLQR-NSIICALVFLVSFVLGDVNIVSPSSKATFSPSGGTVSV-PVEWMDNGAYPS--LSK
Knh1p     MLIVLFLTLFCSVVFRTAYC--DVAIVAPEPNSVYDLSGTSQAVVKVKWMHTDNTPQEKDFVR


                    70        80        90       100       110       120
                    |         |         |         |         |         |
CaKre9p   AKGYTISLCTGPTSDGDIQCLDPL---VKNEAIAGKSK---TVSIPQNSVPNGYYYFQIYVT
Kre9p     ISTFTFSLCTGP--NNNIDCVAVLASKITPSELTQDDKVYSYTAEFASTLTGNGQYYIQVFAQ
Knh1p     --YTFTLCSG--TNAMIEAMATLQT-LSASDLTDNE---FNAIIENTVGTDGVYFIQVFAQ


                   130       140       150       160       170       180
                    |         |         |         |         |         |
CaKre9p   FTNGGTTIHYSPRFKLTGMSGPTATLDVTETGSVPADQASGFDT----ATTADSKSFTVPYTL
Kre9p     VDGQGYTIHYTPRFQLTSMGGVTAYTYSATTEPTPQTSIQTTTTNNAQATTIDSRSFTVPYTK
Knh1p     -TAIGYTIHYTNRFKLKGMIGTKA---ANPSMITIAPEAQTRITTGDVGATIDSKSFTVPYNL
```

FIG. 2A

```
        190       200       210       220       230       240       250
         |         |         |         |         |         |         |

CaKre9p  QTGKTRYAPMQMQPGTKVTATTWSMKFPTSAVTYYSTKAGTPNVASTITPGWSYTAESAVNYA
Kre9p    QTGTSRFAPMQMQPNTKVTATTWTRKFATSAVTYYSTFGSLPEQATTITPGWSYTISSGVNYA
Knh1p    QTGVVKYAPMQLQPATKVTAKTWKRKYATSEVTYYTLRNSVDQHTTVTPGWSYIITADSNYA

        260       270       280
         |         |         |

CaKre9p  SVAPYPTY---WYPASERVSKATISAATKRRRWLD
Kre9p    TPASMPSDNGGWYKPSKRLSLS----ARKINMRKV
Knh1p    T-APMPADNGGWYNPRKRLSLTARKVNALRHR
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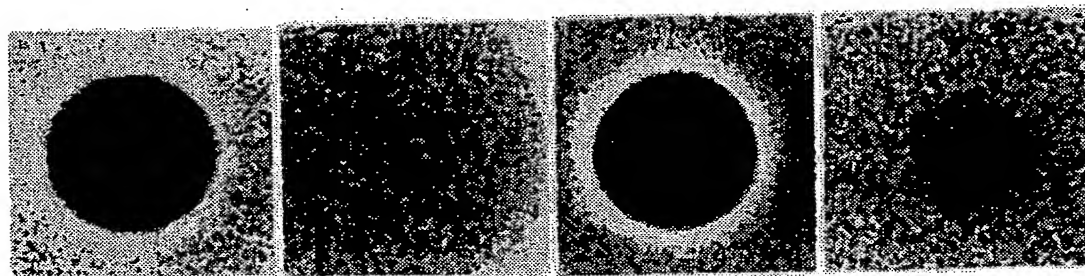
FIG. 2B

FIG. 3B

WT

Δkre9

Δkre9
+YEp352-KRE9

Δkre9
+YEp352-CaKRE9

FIG. 3A

WT

Δkre9

Δkre9
+YEp352-KRE9

Δkre9
+YEp352-CaKRE9

DraI   SalI   BstxI   BamHI   SspI DraI

405 bp caKRE9 probe

caKRE9

DraI   SalI   (BstxI)   BamHI   SspI DraI

hisG   caURA3   hisG

(bglII)

BamHI

FIG. 4A

Fig. 4B

8 / 8

| 1 | 2 | 3 | 4 | 5 | 6 |
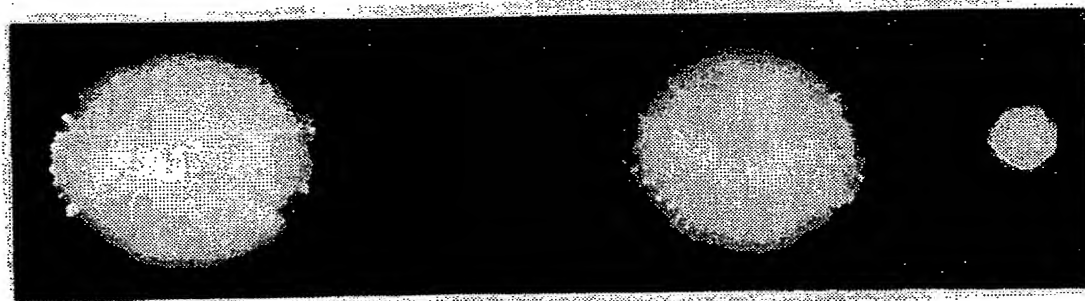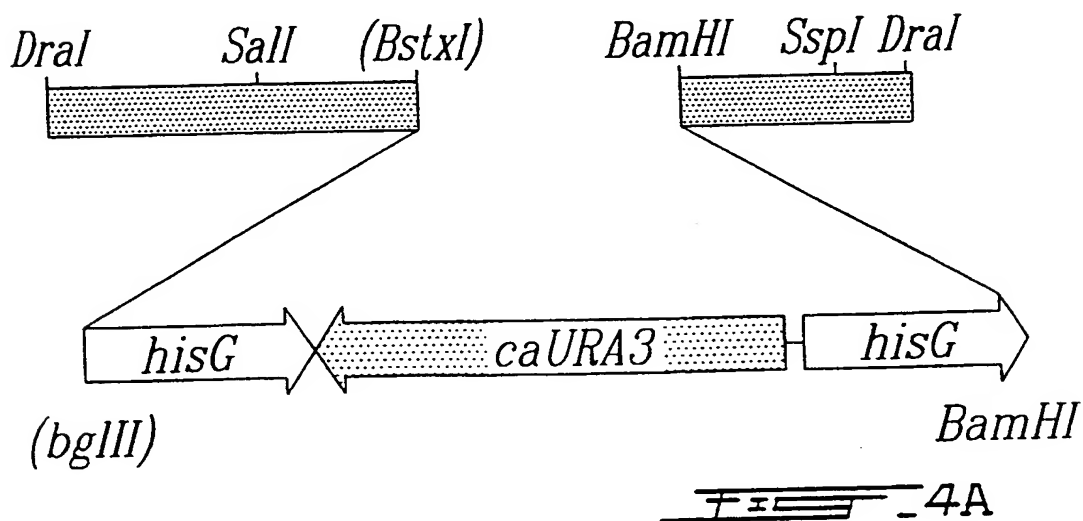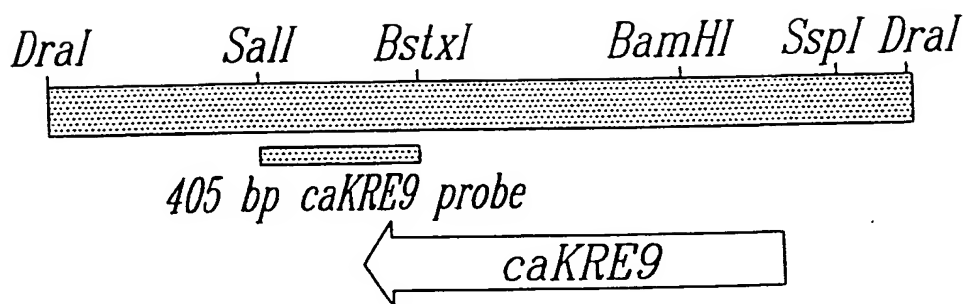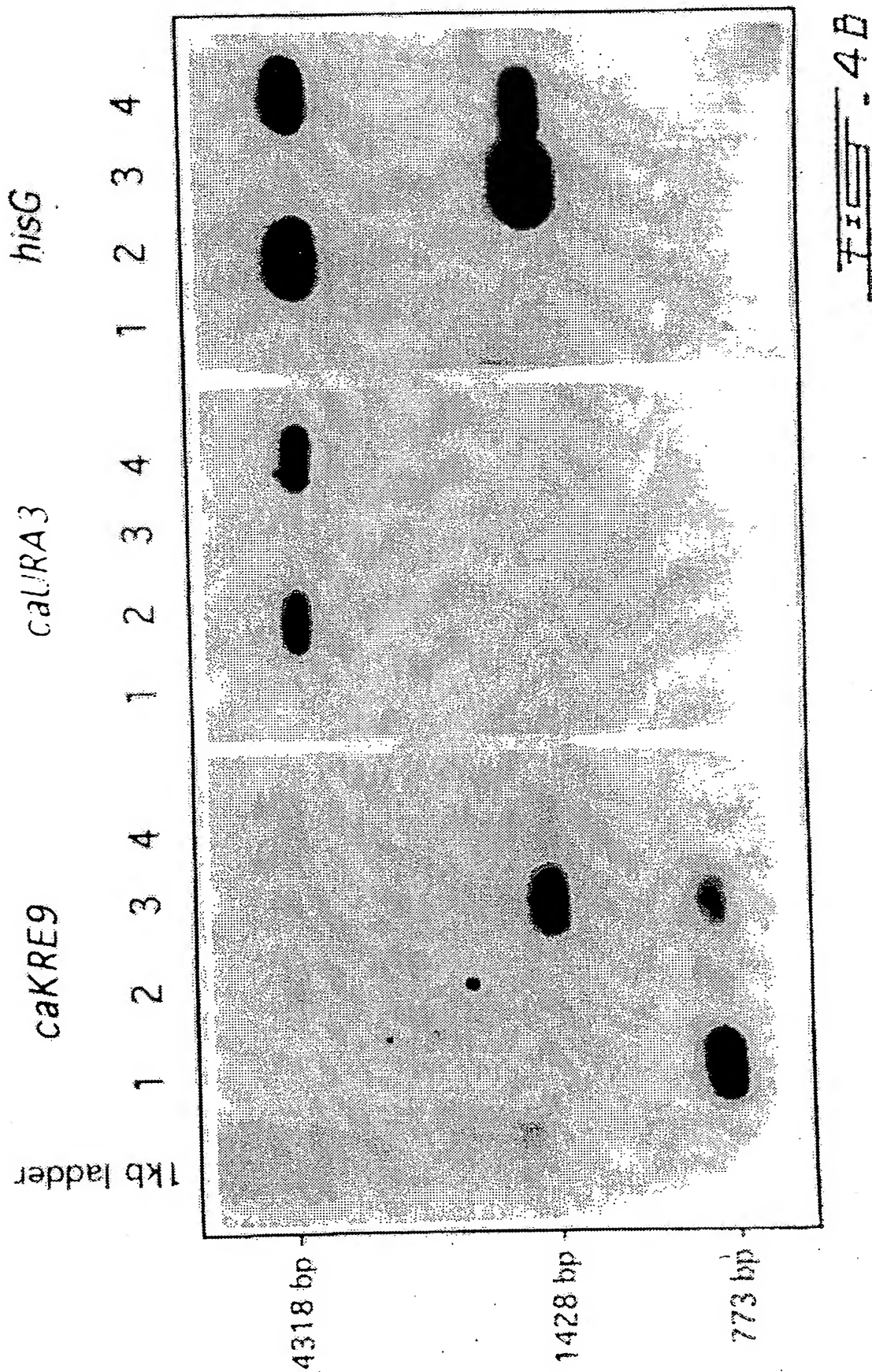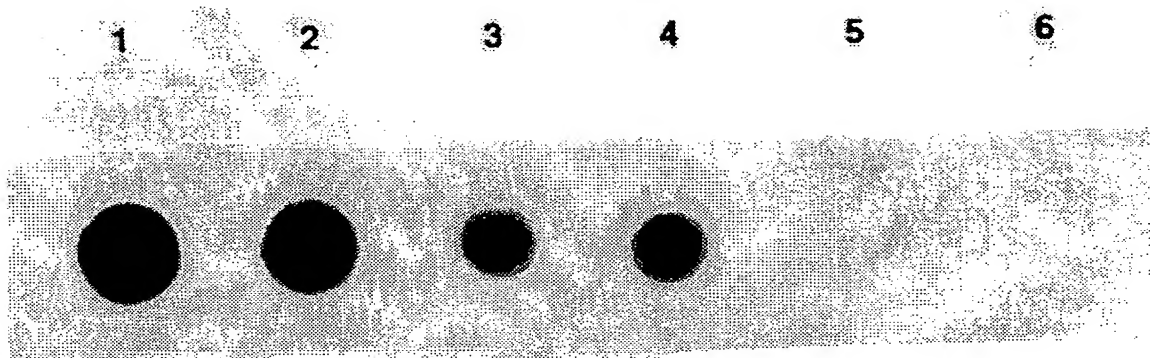
1, 2:   **Wild type**

3, 4:   *CaKRE9*
        Cakre9::hisG

5, 6:   *Cakre9::hisG*
        Cakre9::hisG-URA3-hisG

FIG. 5

SEQUENCE LISTING

<110> McGILL UNIVERSITY
      BUSSEY, Howard
      LUSSIER, Marc
      SDICU, Anne-Marie
      SHAHINIAN, Sarkis, Serge

<120> NEW CANDIDA ALBICANS KRE9 AND USES
      THEREOF

<130> 1770-195PCT   FC/ld

<150> CA 2,218,446
<151> 1997-12-12

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gcctctacta ttaccccagg ttggagttat actgctgaat ctgccgttaa ctatgctagt   720
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                20                  25                  30
Thr Phe Ser Gly Ser Ser Gly Ser Ala Ser Ile Lys Ile Thr Trp Asp
            35                  40                  45
Asp Ser Asp Asp Ser Asp Ser Pro Lys Ser Leu Asp Asn Ala Lys Gly
        50                  55                  60
```

```
Tyr Thr Ile Ser Leu Cys Thr Gly Pro Thr Ser Asp Gly Asp Ile Gln
65              70                  75                  80
Cys Leu Asp Pro Leu Val Lys Asn Glu Ala Ile Ala Gly Lys Ser Lys
            85                  90                  95
Thr Val Ser Ile Pro Gln Asn Ser Val Pro Asn Gly Tyr Tyr Tyr Phe
            100                 105                 110
Gln Ile Tyr Val Thr Phe Thr Asn Gly Gly Thr Thr Ile His Tyr Ser
        115                 120                 125
Pro Arg Phe Lys Leu Thr Gly Met Ser Gly Pro Thr Ala Thr Leu Asp
        130             135                 140
Val Thr Glu Thr Gly Ser Val Pro Ala Asp Gln Ala Ser Gly Phe Asp
145             150                 155                 160
Thr Ala Thr Thr Ala Asp Ser Lys Ser Phe Thr Val Pro Tyr Thr Leu
            165                 170                 175
Gln Thr Gly Lys Thr Arg Tyr Ala Pro Met Gln Met Gln Pro Gly Thr
            180                 185                 190
Lys Val Thr Ala Thr Thr Trp Ser Met Lys Phe Pro Thr Ser Ala Val
        195                 200                 205
Thr Tyr Tyr Ser Thr Lys Ala Gly Thr Pro Asn Val Ala Ser Thr Ile
        210                 215                 220
Thr Pro Gly Trp Ser Tyr Thr Ala Glu Ser Ala Val Asn Tyr Ala Ser
225             230                 235                 240
Val Ala Pro Tyr Pro Thr Tyr Trp Tyr Pro Ala Ser Glu Arg Val Ser
            245                 250                 255
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            20                  25                  30
Thr Phe Ser Pro Ser Gly Gly Thr Val Ser Val Pro Val Glu Trp Met
        35                  40                  45
Asp Asn Gly Ala Tyr Pro Ser Leu Ser Lys Ile Ser Thr Phe Thr Phe
    50                  55                  60
Ser Leu Cys Thr Gly Pro Asn Asn Asn Ile Asp Cys Val Ala Val Leu
65              70                  75                  80
Ala Ser Lys Ile Thr Pro Ser Glu Leu Thr Gln Asp Asp Lys Val Tyr
            85                  90                  95
Ser Tyr Thr Ala Glu Phe Ala Ser Thr Leu Thr Gly Asn Gly Gln Tyr
            100                 105                 110
Tyr Ile Gln Val Phe Ala Gln Val Asp Gly Gln Gly Tyr Thr Ile His
        115                 120                 125
Tyr Thr Pro Arg Phe Gln Leu Thr Ser Met Gly Gly Val Thr Ala Tyr
        130             135                 140
Thr Tyr Ser Ala Thr Thr Glu Pro Thr Pro Gln Thr Ser Ile Gln Thr
145             150                 155                 160
Thr Thr Thr Asn Asn Ala Gln Ala Thr Thr Ile Asp Ser Arg Ser Phe
            165                 170                 175
Thr Val Pro Tyr Thr Lys Gln Thr Gly Thr Ser Arg Phe Ala Pro Met
            180                 185                 190
```

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        195                 200                 205
Phe Ala Thr Ser Ala Val Thr Tyr Tyr Ser Thr Phe Gly Ser Leu Pro
        210                 215                 220
Glu Gln Ala Thr Thr Ile Thr Pro Gly Trp Ser Tyr Thr Ile Ser Ser
225                         230                 235                 240
Gly Val Asn Tyr Ala Thr Pro Ala Ser Met Pro Ser Asp Asn Gly Gly
                245                 250                 255
Trp Tyr Lys Pro Ser Lys Arg Leu Ser Leu Ser Ala Arg Lys Ile Asn
                260                 265                 270
Met Arg Lys Val
        275


        <210> 4
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                20                  25                  30
Tyr Asp Leu Ser Gly Thr Ser Gln Ala Val Val Lys Val Lys Trp Met
        35                  40                  45
His Thr Asp Asn Thr Pro Gln Glu Lys Asp Phe Val Arg Tyr Thr Phe
        50                  55                  60
Thr Leu Cys Ser Gly Thr Asn Ala Met Ile Glu Ala Met Ala Thr Leu
65                  70                  75                  80
Gln Thr Leu Ser Ala Ser Asp Leu Thr Asp Asn Glu Phe Asn Ala Ile
                85                  90                  95
Ile Glu Asn Thr Val Gly Thr Asp Gly Val Tyr Phe Ile Gln Val Phe
                100                 105                 110
Ala Gln Thr Ala Ile Gly Tyr Thr Ile His Tyr Thr Asn Arg Phe Lys
        115                 120                 125
Leu Lys Gly Met Ile Gly Thr Lys Ala Ala Asn Pro Ser Met Ile Thr
        130                 135                 140
Ile Ala Pro Glu Ala Gln Thr Arg Ile Thr Thr Gly Asp Val Gly Ala
145                 150                 155                 160
Thr Ile Asp Ser Lys Ser Phe Thr Val Pro Tyr Asn Leu Gln Thr Gly
                165                 170                 175
Val Val Lys Tyr Ala Pro Met Gln Leu Gln Pro Ala Thr Lys Val Thr
                180                 185                 190
Ala Lys Thr Trp Lys Arg Lys Tyr Ala Thr Ser Glu Val Thr Tyr Tyr
        195                 200                 205
Tyr Thr Leu Arg Asn Ser Val Asp Gln His Thr Thr Val Thr Pro Gly
        210                 215                 220
Trp Ser Tyr Ile Ile Thr Ala Asp Ser Asn Tyr Ala Thr Ala Pro Met
225                 230                 235                 240
Pro Ala Asp Asn Gly Gly Trp Tyr Asn Pro Arg Lys Arg Leu Ser Leu
                245                 250                 255
Thr Ala Arg Lys Val Asn Ala Leu Arg His Arg
                260                 265
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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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(54) Title: NEW *CANDIDA ALBICANS* KRE9 AND USES THEREOF

(57) Abstract

The present invention relates to an isolated DNA which codes for a gene essential for cell wall glucan synthesis of *Candida albicans*, wherein the gene is referred to as *CaKRE9*, wherein the sequence of the DNA is as set forth in Fig. 1. The present invention relates to antifungal *in vitro* and *in vivo* screening assays for identifying compounds which inhibit the synthesis, assembly and/or regulation of β1,6–glucan. There is also disclosed an *in vitro* method for the diagnosis of diseases caused by fungal infection in a patient.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12Q   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BROWN AND BUSSEY: "THE YEAST KRE9 GENE ENCODES AN O GLYCOPROTEIN INVOLVED IN CELL SURFACE BETA-GLUCAN ASSEMBLY" MOLECULAR AND CELLULAR BIOLOGY, vol. 13, no. 10, 1993, pages 6346-6356, XP002104903 see the whole document ---	1-10
Y	US 5 194 600 A (BUSSEY HOWARD ET AL) 16 March 1993 See examples III-VI see the whole document --- -/--	1-10

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
4 June 1999	16/06/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	BOONE ET AL.: "ISOLATION FROM CANDIDA ALBICANS OF A FUNCTIONAL HOMOLOG OF THE SACCHAROMYCES CEREVISIAE KRE1 GENE, WHICH IS INVOLVED IN CELL WALL BETA-GLUCAN SYNTHESIS" JOURNAL OF BACTERIOLOGY, vol. 173, no. 21, 1991, pages 6859-6864, XP002104956 see the whole document ---	
A	DIJKGRAAF ET A.: "THE KNH1 GENE OF SACCHAROMYCES CEREVISIAE IS A FUNCTIONAL HOMOLG OF KRE9" YEAST, vol. 12, 1996, pages 683-692, XP002104904 see the whole document ---	
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